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Astrocyte-specific expression of hamster prion protein (PrP) renders PrP knockout mice susceptible to hamster scrapie

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Transmissible spongiform encephalopathies are characterized by spongiosis, astrogliosis and accumulation of PrP^{Sc}, an isoform of the normal host protein PrP^C. The exact cell types responsible for agent propagation and pathogenesis are still uncertain. To determine the possible role of astrocytes, we generated mice devoid of murine PrP but expressing hamster PrP transgenes driven by the astrocyte-specific GFAP promoter. After inoculation with hamster scrapie, these mice accumulated infectivity and PrP^{Sc} to high levels, developed severe disease after 227 ± 5 days and died 7 ± 4 days later. Therefore, astrocytes could play an important role in scrapie pathogenesis, possibly by an indirect toxic effect on neurons. Interestingly, mice expressing the same transgenes but also endogenous murine PrP genes propagated infectivity without developing disease.

Keywords: astrocytes/hamster scrapie prions/PrP/transgenic mice

Introduction

Transmissible spongiform encephalopathies (TSEs) are degenerative disorders of the central nervous system (CNS) which occur naturally in man and in a variety of other animals. They include scrapie of sheep and goats, bovine spongiform encephalopathy (BSE) in cattle and several human diseases such as Creutzfeldt–Jakob disease (CJD), Kuru, Gerstmann–Sträussler–Scheinker syndrome (GSS) and fatal familial insomnia (FFI) (Pocchiari, 1994;

Prusiner, 1996; Caughey and Chesebro, 1997). Since its first recognition in 1986, BSE has acquired epidemic proportions in the UK (Wilesmith, 1992), and the recent appearance of a new variant of CJD (vCJD) has raised concern that BSE might be communicable to humans (Will *et al.*, 1996). One of the hallmarks of TSE is the accumulation in the brain of a protease-resistant protein, PrP^{Sc} or PrP-res, which is derived by a poorly understood post-translational event from a normal host-encoded protease-sensitive isoform, designated PrP^C or PrP-sen (Chesebro *et al.*, 1985; Oesch *et al.*, 1985; Basler *et al.*, 1986). PrP^C is attached by a glycolipid anchor to the cell surface (Stahl *et al.*, 1987) and is expressed by many cell types, including neurons (Kretzschmar *et al.*, 1986), astrocytes (Moser *et al.*, 1995; van Keulen *et al.*, 1995), lymphocytes (Cashman *et al.*, 1990), follicular dendritic cells (McBride *et al.*, 1992) and tumor cell lines of various lineages (Caughey *et al.*, 1988). Brain-derived preparations highly enriched for scrapie infectivity contain PrP^{Sc} as a major protein component (Bolton *et al.*, 1982), but no scrapie-specific nucleic acid has been detected (Kellings *et al.*, 1993). Prusiner suggested that the etiologic agent of TSE might be PrP^{Sc} itself (Prusiner, 1982) and that agent propagation might occur by a mechanism of PrP^{Sc} induced conformational transition of PrP^C into new PrP^{Sc} molecules (Prusiner, 1989, 1991, 1993; Caughey *et al.*, 1991; Pan *et al.*, 1993).

Mice devoid of functional PrP genes are resistant to scrapie and unable to propagate the agent (Büeler *et al.*, 1993; Sailer *et al.*, 1994). Efficient cross-species transmission of TSE is dependent on expression of PrP from the same species as the source of the challenge TSE agent (Scott *et al.*, 1989; Prusiner *et al.*, 1990). Introduction of hamster PrP (HaPrP) transgenes into PrP null mice restores susceptibility to hamster scrapie but not to mouse scrapie, and vice versa (Büeler *et al.*, 1993; Fischer *et al.*, 1996).

The cell types involved in agent propagation have not been completely identified. Some data indicate that in the spleen (where in most models of scrapie, accumulation of scrapie agent is first detected), the follicular dendritic cells constitute the pathogenetic compartment (Kitamoto *et al.*, 1991; McBride *et al.*, 1992). It is generally assumed that agent propagation and pathology in the brain is neuron-dependent, and it was recently shown that transgenic mice expressing a hamster PrP minigene under the control of the neuron-specific enolase (NSE) promoter are highly susceptible to the hamster scrapie agent (Race *et al.*, 1995). In these mice, HaPrP expression was found exclusively in neurons and not in glial cells or cells within the spleen or lymph nodes. Thus, neuron-specific PrP expression is sufficient to sustain scrapie infection, and PrP expression in non-neuronal cells, in particular astrocytes and cells of the lymphoreticular system, is not required, at least in the case of intracerebral inoculation.

The possibility that astrocytes might also contribute to the natural disease process is suggested by the findings that astrocytes express PrP (Moser *et al.*, 1995), and that in at least one model they are also the earliest site of PrP^{Sc} accumulation in the brain (Diedrich *et al.*, 1991). Here we show that PrP knockout mice transgenic for an HaPrP gene under control of the glial fibrillary acidic protein (GFAP) gene promoter express PrP predominantly if not exclusively in astrocytes, and are susceptible to intracerebral inoculation with hamster scrapie agent. These mice accumulate protease-resistant HaPrP and high titers of hamster scrapie agent in their brains, and show pathological changes similar to those of non-transgenic wild-type mice inoculated with mouse scrapie. Interestingly, mice expressing the same transgenes as well as endogenous murine PrP genes propagated infectivity without developing disease.

Results

Generation and characterization of GFAP-HaPrP transgenic mice

To investigate whether astrocyte-restricted expression of HaPrP suffices to render mice susceptible to hamster scrapie agent, transgenic mice were generated in which a 1 kb cDNA encompassing the entire HaPrP open reading frame was expressed under control of the glial fibrillary acidic protein (GFAP) promoter (Johnson *et al.*, 1995) (Figure 1A). Transgene-positive founder mice were bred to C57Bl/10 mice and positive F₁ progeny were identified. Among five independent founder lines, *Tg3* mice showed the highest level of PrP expression in the brain (Figure 1B). These mice expressed HaPrP mRNA in the brain (Figure 1C). Western analysis revealed HaPrP protein in the brain but not in spleen, heart, lung, thymus, muscle, liver or kidney (Figure 1D). *Tg3* mice were crossed with PrP null (*Prnp*^{0/0}) mice (Büeler *et al.*, 1992) to yield *Tg3/Prnp*^{0/+} offspring which were intercrossed to produce *Tg3/Prnp*^{0/0} mice. In initial experiments, the HaPrP mRNA expression level in the brains of these mice was below detectability by *in situ* hybridization. Therefore, we examined the cell specificity of HaPrP expression after cryolesion-mediated upregulation of GFAP-directed transgene expression (Johnson *et al.*, 1995). Brain sections from *Tg3/Prnp*^{0/0} mice sacrificed 48 h after cryolesioning were subjected to *in situ* hybridization for HaPrP followed by immunohistochemistry for GFAP. As shown in Figure 2G, cells in the vicinity of the lesion that contained PrP mRNA also expressed GFAP. In GFAP-positive cells of the hippocampus (Figure 2D), PrP mRNA was expressed to a lesser extent, perhaps because of its distance from the lesioned site or because GFAP protein is more stable than PrP mRNA. For comparison, Figure 2 shows that in the hippocampal formation and also in the cerebral cortex of *Prnp*^{+/+} mice, neurons were strongly positive for PrP mRNA, while no PrP transcripts were detected in brains of non-transgenic *Prnp*^{0/0} mice (compare panels F and I with E and H). These findings indicated that in the brain, the GFAP-HaPrP transgene was expressed only in astrocytes. Experiments were then initiated to study the susceptibility of these mice to hamster scrapie agent.

Susceptibility of GFAP-HaPrP transgenic mice to hamster scrapie

When *Tg3* mice and non-transgenic littermates were inoculated with hamster scrapie agent (strain 263K), neither group developed disease by as late as 750 days after inoculation, whereas all hamsters which were similarly inoculated died of scrapie by 77 ± 3 days (Figure 3). In contrast, when *Tg3/Prnp*^{0/0} mice were challenged with hamster scrapie, the mice developed severe scrapie symptoms consisting of ataxia, paralysis, kyphosis, foot clasp reflex, mincing gait and disorientation around 227 ± 5 days, and died at 233 ± 3 days post-inoculation. Interestingly, disease in these mice presented with a short clinical disease period of only 7 ± 4 days, as compared with 13 ± 11 days for wild-type (CD-1) mice inoculated with RML mouse scrapie (Büeler *et al.*, 1994), suggesting unusual kinetics of the onset and development of pathogenic processes. In a second experiment, the *Tg3* transgenes were introduced into PrP knockout mice on a different genetic background (129/Ola) (Manson *et al.*, 1994a), to yield *RML-Tg3/Prnp*^{-/-} mice. After inoculation with hamster scrapie agent (strain 263K), 9 out of 12 mice developed disease and died as in the previous experiment, one animal developed disease 462 days after inoculation and two mice which did not show clinical symptoms were sacrificed 564 days after inoculation and found to have high levels of PrP^{Sc} in their brains. Thus, in almost all cases, expression of HaPrP in astrocytes was sufficient to confer susceptibility to clinical disease in *Prnp*^{0/0} mice, but not in *Prnp*^{+/+} mice. These results demonstrated the profound influence of endogenous murine PrP^C on the susceptibility of HaPrP-transgenic mice to hamster scrapie agent; similar interference of endogenous murine PrP with pathogenicity of TSE agents from other species has been described previously (Scott *et al.*, 1989; Race *et al.*, 1995; Telling *et al.*, 1996).

Protease-resistant HaPrP in transgenic GFAP-HaPrP mice

One of the hallmarks of TSE diseases is the accumulation in the brain of a protease-resistant isoform of PrP termed PrP^{Sc}. We assessed the formation of PrP^{Sc} in scrapie-infected transgenic mice by Western immunoblot analysis of brain extracts using the HaPrP-specific monoclonal antibody 3F4 (Kascsak *et al.*, 1987). Although the level of hamster PrP^C in uninfected *Tg3/Prnp*^{0/0} mice was only about 5% of the level detected in normal hamster brain (Figure 4A, lanes 1 and 5), terminally ill scrapie-inoculated *Tg3/Prnp*^{0/0} mice showed similar high levels of protease-resistant PrP to clinically ill hamsters (Figure 4B). The inordinately increased level of total HaPrP in scrapie-infected as compared with uninfected *Tg3/Prnp*^{0/0} mice (Figure 4A, compare lanes 1 and 3) could be due to increased expression of HaPrP as a consequence of astrogliosis, in addition to the accumulation of HaPrP^{Sc}. Northern blot analysis of poly(A)⁺ RNA showed a 2-fold increase in HaPrP transcript level in the brains of *Tg3/Prnp*^{0/0} mice in response to scrapie infection, in contrast to the levels in wild-type mice which were not altered by scrapie infection (Figure 4C, compare lanes 5 and 6 with 7 and 8).

Surprisingly, asymptomatic *Tg3/Prnp*^{+/+} mice sacrificed at 300, 630 or 750 days post-inoculation also had detectable

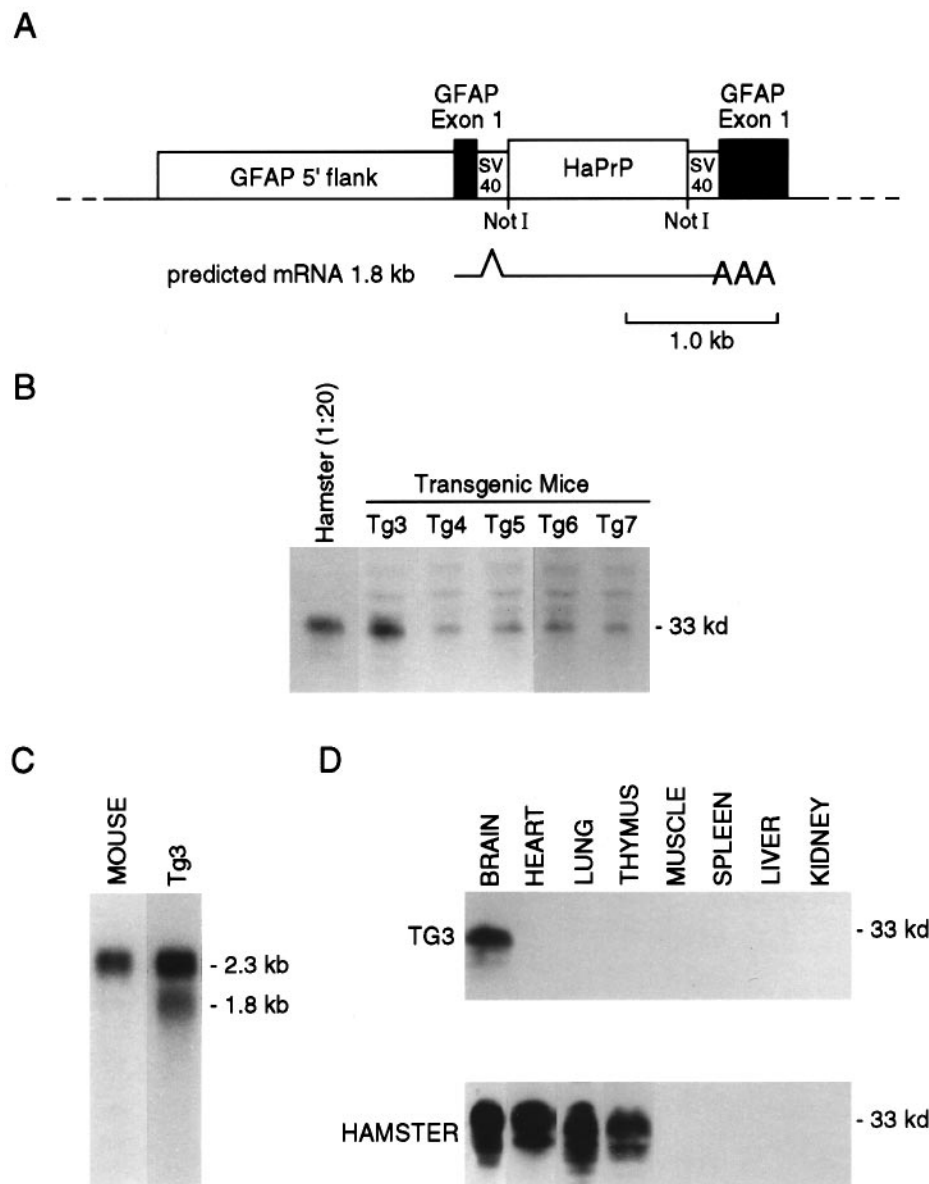


Fig. 1. Structure of the GFAP-HaPrP transgene and characterization of the transgenic mice. **(A)** Partial structure of the GFAP-HaPrP transgene and predicted mRNA. A 1.2 kb fragment of hamster PrP (HaPrP) cDNA (Robakis *et al.*, 1986) containing the open reading frame, some untranslated PrP sequences and Bluescript polylinker sequences was used to replace the *lacZ* gene between the *NotI* sites in clone C-445 (Johnson *et al.*, 1995). This construct contained ~2 kb of GFAP 5' flanking sequences, 89 residues of GFAP exon1, 0.2 kb of SV40 DNA with splice sites, the 1.2 kb HaPrP insert, 0.2 kb of SV40 DNA with a poly(A) signal sequence, followed by the remainder of the GFAP gene. This construct was expected to produce a messenger RNA of 1.8 kb, as shown. **(B)** Analysis of HaPrP^C in brain by immunoblotting. Brain homogenates were prepared from uninfected hamsters and F₁ crosses of GFAP-HaPrP transgenic founder mice with C57BL/10 mice and were analyzed by immunoblotting with HaPrP-specific monoclonal antibody 3F4. **(C)** Analysis of PrP mRNA in brain. mRNA from normal and Tg3 mouse brain was analyzed by Northern blotting as described in Materials and methods. A 2.3 kb mRNA representing the normal mouse PrP transcript was seen in both samples, and a 1.8 kb mRNA corresponding to the predicted transcript from the GFAP-HaPrP transgene was detected in Tg3 mice. **(D)** Immunoblot analysis of HaPrP^C in various tissues of hamster and Tg3 transgenic mice using monoclonal antibody 3F4. Each lane contained protein derived from 5 mg of tissue.

PrP^{Sc}, but quantitation by serial dilution showed that its level was at least 100-fold lower than that observed in terminally ill hamsters or *Tg3/Prnp*^{0/0} mice (Figure 4B). A correlation between PrP^{Sc} level and clinical disease is, however, not a general finding (Büeler *et al.*, 1994; Collinge *et al.*, 1995; Telling *et al.*, 1996).

Propagation of hamster scrapie agent in transgenic GFAP-HaPrP mice

To determine whether hamster scrapie agent was propagated in the brains of asymptomatic *Tg3/Prnp*^{+/+} mice

or non-transgenic littermates, brain homogenates from animals sacrificed at various times after inoculation were injected intracerebrally into hamsters. Recipients of brain homogenate from *Tg3/Prnp*^{+/+} mice died after 90–95 days, which corresponds to a titer of $\sim 2 \times 10^7$ – 2×10^8 LD₅₀ units/g of brain tissue, whereas samples from clinically ill *Tg3/Prnp*^{0/0} mice had 2×10^9 – 2×10^{10} LD₅₀ units/g (Table I). In contrast, non-transgenic control mice had much less hamster scrapie infectivity, corresponding to a level of 20–2000 LD₅₀ units/g. Thus, the presence of normal mouse PrP reduced formation of HaPrP^{Sc} and

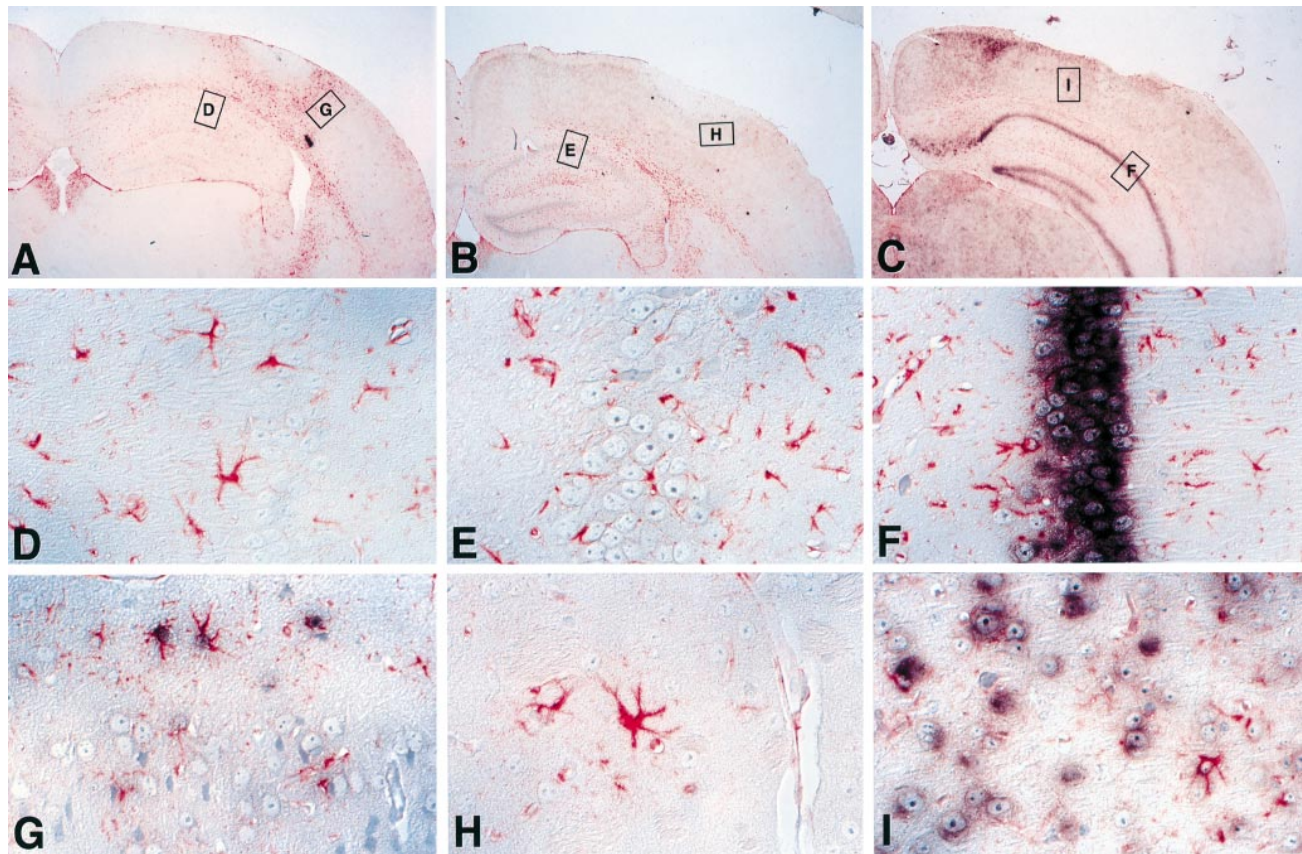


Fig. 2. Brain sections doubly labeled for PrP mRNA and GFAP. Double labeling of *Prnp* mRNA (black) and GFAP (red) on sections of cryolesioned brain of *Tg3/Prnp*^{0/0} mice (left column), *Prnp*^{0/0} mice (middle column) and wild-type mice (right column) 48 h after cryolesioning (Phillips *et al.*, 1995) of the right frontoparietal cortex (duration 60 s). At low power magnification (A, B and C), the necrotic zone and the penumbra of the cryolesion appear demarcated by reactive astrocytes. Labeled rectangles indicate the detailed view of the respective area, hippocampus or penumbra zone of the cortex. In *Tg3/Prnp*^{0/0} mice, coexpression of HaPrP mRNA (black) and GFAP (red) is visible after strong activation of the GFAP promoter in the penumbra zone of the cryolesion (G), while HaPrP mRNA is barely detectable in resting astrocytes (D). *Prnp*^{0/0} mice show activated astrocytes around cryolesioned areas (H) and resting astrocytes in the hippocampus (E), but no PrP mRNA. Wild-type mice show PrP mRNA in hippocampal (F) and cortical neurons (I) but not in activated (I) or resting (F) astrocytes.

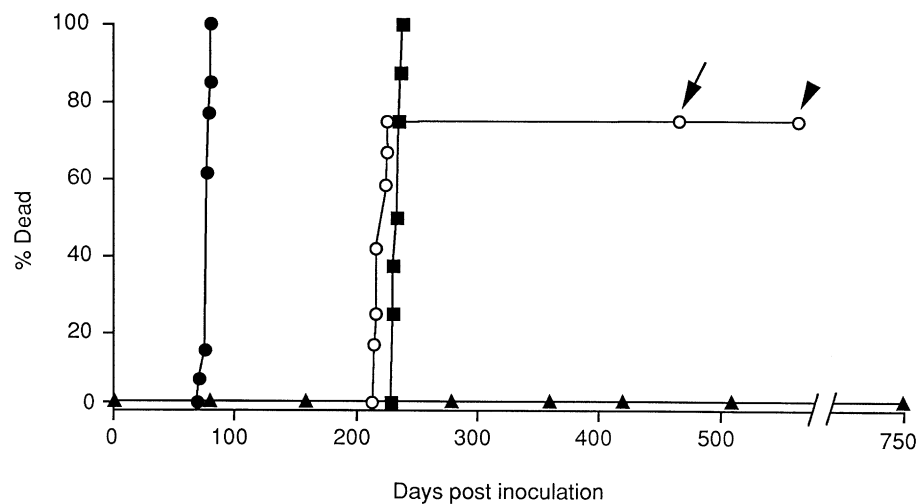


Fig. 3. Kinetics of death due to scrapie of animals inoculated intracerebrally with hamster scrapie agent. (●) Hamsters ($N = 13$) inoculated with strain 263K. (○) RML-Tg3/*Prnp*^{-/-} mice ($N = 12$) inoculated with strain 263K. One mouse developed scrapie-like disease 462 days post-inoculation (arrow) and was sacrificed 564 days after inoculation. No PrP^{Sc} was detected in the brain of this mouse. Two mice were sacrificed 564 days after inoculation (arrowhead). Both of these mice had PrP^{Sc} levels in their brains similar to a scrapie sick hamster. One of these mice was listless and ataxic; this same mouse had collapsed cerebral hemispheres at necropsy. The other mouse was listless but did not show any other clinical symptoms. (■) Zürich-Tg3/*Prnp*^{0/0} mice ($N = 8$) inoculated with strain Sc237. (▲) Non-transgenic mice C57BL/10 ($N = 10$) inoculated with 263K showed no clinical disease during the period of observation (750 days). 'Death times' represent interval to a moribund condition or in a few cases, to actual death.

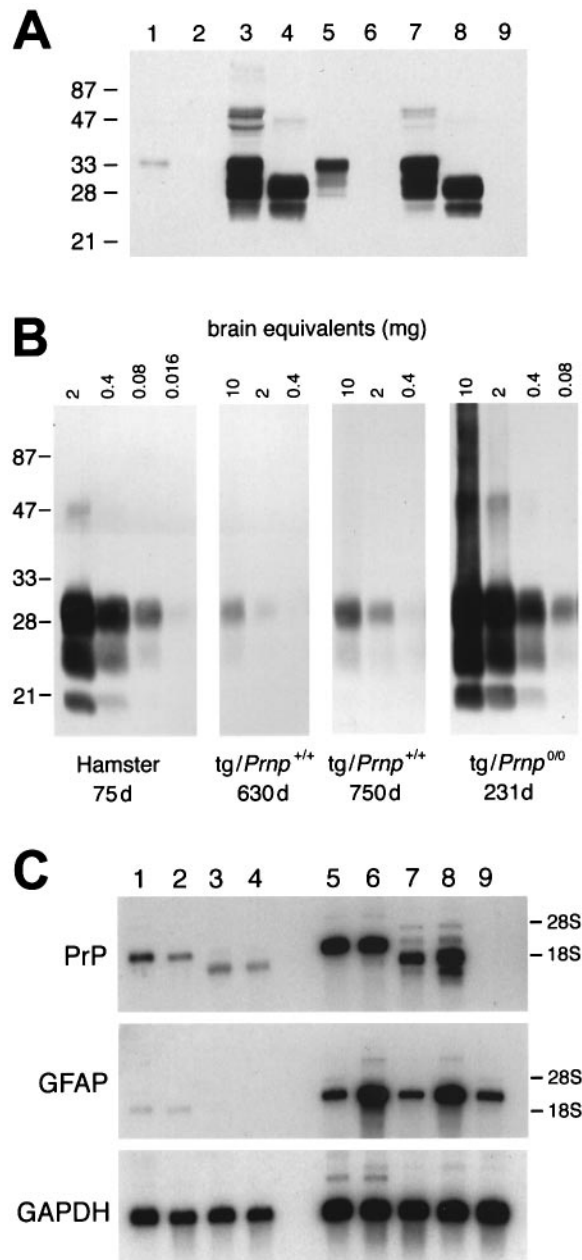


Fig. 4. Detection of HaPrP^{Sc} and PrP mRNA in organ homogenates. (A) Western analysis of proteinase K-digested samples (even-numbered lanes) and untreated samples (odd-numbered lanes). Lanes 1–4, *Tg3/Prnp*^{0/0} mouse uninoculated (lanes 1 and 2) or inoculated with Sc237 hamster scrapie agent and terminally scrapie sick (lanes 3 and 4). Lanes 5–8, hamster uninoculated (lanes 5 and 6) or inoculated and terminally scrapie sick (lanes 7 and 8). Lane 9, *Prnp*^{0/0} mouse, not inoculated. Molecular weight markers are depicted on the left with molecular masses expressed in kDa. (B) Western analysis of serial 5-fold dilutions of proteinase K-treated brain extracts from hamsters [75 days post-inoculation (d)], *Tg3 (Prnp*^{+/+}) mice (630 d and 750 d), and from *Tg3/Prnp*^{0/0} (231 d). Molecular weight markers are indicated on the left with molecular masses expressed in kDa. (C) Northern analysis of poly(A)⁺ RNA (equivalent of 50 µg of total RNA per lane) in spleens (lanes 1–4) and brains (lanes 5–9) of wild-type mice uninoculated (lanes 1 and 5) or inoculated with RML mouse scrapie agent and terminally scrapie sick (lanes 2 and 6) and *Tg3/Prnp*^{0/0} mice uninoculated (lanes 3 and 7) or inoculated with Sc237 hamster scrapie agent and terminally sick (lanes 4 and 8). Lane 9, brain of *Prnp*^{0/0}, not inoculated. After autoradiography the filter was stripped and successively hybridized with a GFAP and a GAPDH probe.

hamster scrapie infectivity and prevented clinical disease in *Tg3/Prnp*^{+/+} mice.

Low levels of hamster scrapie infectivity were detected in the spleen of a *Tg3/Prnp*^{0/0} mouse sacrificed 233 days after intracerebral (i.c.) inoculation. This could either be due to transport of residual inoculum or of scrapie agent formed in the CNS to the lymphoreticular system, or represent propagation of infectivity in the spleen. The latter interpretation, if correct, could be explained by the presence of low levels of HaPrP mRNA in the spleens of both uninfected and scrapie-infected *Tg3/Prnp*^{0/0} mice. The GFAP promoter is therefore active in spleen tissue, as shown by the presence of low levels of GFAP mRNA in wild-type spleen (Figure 4C, lanes 1 and 2) and in *Tg3/Prnp*^{0/0} spleen (lanes 3 and 4, visible on the original film but not in the photograph).

Neuropathology in scrapie-inoculated mice

In the brains of clinically ill *Tg3/Prnp*^{0/0} mice inoculated with hamster agent, spongiform changes and pronounced astrogliosis were found predominantly in the neocortex and hippocampus, with no significant difference between *Tg3/Prnp*^{0/0} transgenic mice inoculated with hamster scrapie and normal mice inoculated with mouse scrapie (Figure 5). Therefore, typical scrapie neuropathology could be observed when PrP was expressed in astrocytes and not in neurons.

Discussion

Spongiosis, astrogliosis and accumulation of PrP^{Sc} are the most prominent neuropathological features associated with TSEs; neuronal loss is not consistently found in mouse scrapie (Scott and Fraser, 1984; S.Brandner, unpublished observations). While neurons are clearly affected in the disease process, it is still unclear whether other brain cells also play a role. Recently it was demonstrated that neuron-specific expression of HaPrP driven by the NSE promoter in transgenic mice is sufficient to mediate susceptibility to hamster scrapie (Race *et al.*, 1995). However, astrocytes may also participate in scrapie pathogenesis because these cells show marked morphological abnormalities early in the disease process (Pattison, 1965; Eklund *et al.*, 1967), and may in some cases be the earliest site of accumulation of PrP^{Sc} in the brain (Diedrich *et al.*, 1991).

In the present paper we report on transgenic mice expressing HaPrP under control of the GFAP promoter. The GFAP expression vector used for generating GFAP-HaPrP transgenic mice was shown previously to confer astrocyte-specific expression upon a *lacZ* reporter transgene (Mucke *et al.*, 1991). In these experiments, *lacZ* expression was restricted to astrocytes in the CNS and lens epithelium, and was not observed in neurons or in other tissues including spleen, lymph node and sciatic nerve. In the GFAP-HaPrP mice described in the present report, HaPrP protein was found only in the brain and was below detection levels in other tissues. Although the constitutive level of HaPrP in the brains of transgenic mice was only ~5% of that observed in normal hamster brain, the GFAP-HaPrP transgene was up-regulated after cryolesioning and after infection with hamster scrapie. Within the limits of detection *in vivo*, HaPrP mRNA co-localized exclusively with GFAP-positive cells in brain

Table I. Bioassay of hamster scrapie infectivity from mouse brains

Donor genotype ^a	Organ	Days p.i.	Log ₁₀ dilution ^b	Days to death ^c	LD ₅₀ /g
<i>Prnp</i> ^{+/+} (non-tg)	brain	300	-2	178 ± 14 (3)	2.10 ¹
<i>Prnp</i> ^{+/+} (non-tg)	brain	630	-2	123 ± 23 (15)	2.10 ³
Tg3/ <i>Prnp</i> ^{+/+}	brain	300	-2	95 ± 3 (10)	2.10 ⁷
Tg3/ <i>Prnp</i> ^{+/+}	brain	630	-2	90 ± 2 (12)	2.10 ⁸
Tg3/ <i>Prnp</i> ^{0/0}	brain	231	-3	83 ± 4 (5)	2.10 ¹⁰
Tg3/ <i>Prnp</i> ^{0/0}	brain	233	-3	90 ± 4 (6)	2.10 ⁹
Tg3/ <i>Prnp</i> ^{0/0}	brain	235	-3	91 ± 5 (6)	2.10 ⁹
Tg3/ <i>Prnp</i> ^{0/0}	spleen	233	-2	212 ± 16 (3)	1–10
Hamster	brain	75	-3	83 ± 2 (6)	2.10 ¹⁰

^a Donor animals were inoculated i.c. with hamster scrapie agent as described in Materials and methods.

^b 10% (w/v) suspensions of tissue in either 0.32 M sucrose or 10 mM Tris-HCl, 5 mM MgCl₂ (= 10⁻¹ dilution) were made, and the dilutions indicated in the table were inoculated i.c. into hamsters.

^c Mean days to death ± standard deviation (number of hamsters inoculated). All inoculated hamsters died of scrapie.

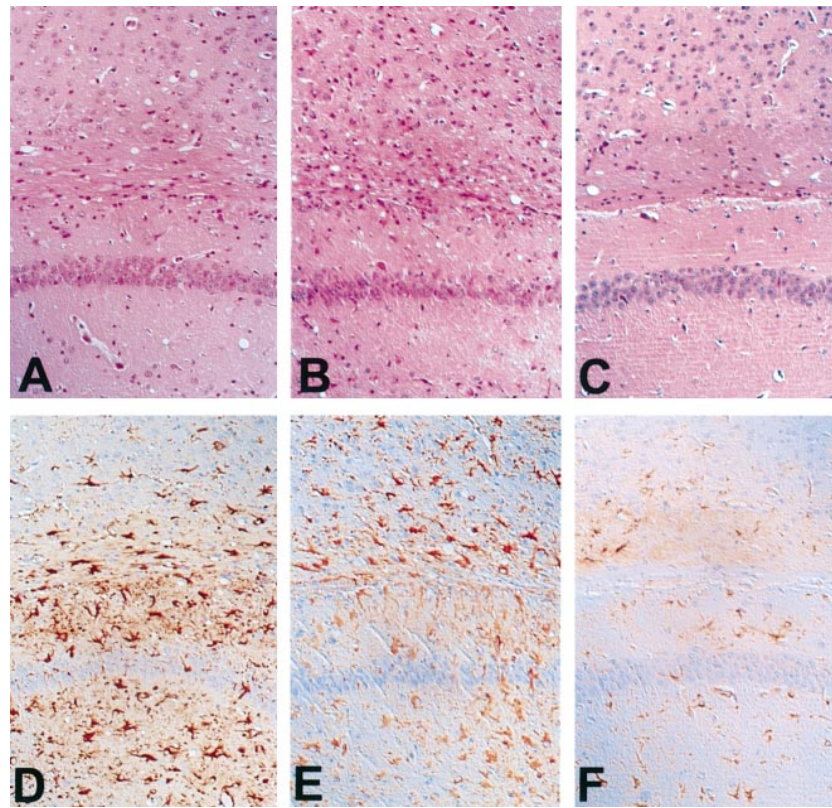


Fig. 5. Neuropathology in terminally ill scrapie-infected mice. Hematoxylin and eosin staining (A–C) and GFAP immunohistochemistry (D–F) of the hippocampus of a Sc237 hamster scrapie-inoculated Tg3/*Prnp*^{0/0} mouse (238 days post-inoculation) (A and D), wild-type mouse inoculated with RML mouse scrapie (166 days post inoculation) (B and E) and RML-inoculated *Prnp*^{0/0} mouse (365 days post inoculation) (C and F). Prominent spongiosis and gliosis were found in the brains of scrapie-infected transgenic and wild-type mice. Histopathological analyses were performed when mice showed clinical symptoms of scrapie. No notable differences in the pathological changes between transgenic and wild-type mice were observed.

sections of Tg3/*Prnp*^{0/0} mice, as expected from the astrocyte specificity of the GFAP promoter (Johnson *et al.*, 1995). There was no evidence for HaPrP expression in neurons, and if any occurred, it would be at very low levels and unlikely to contribute to pathogenesis. No HaPrP protein was seen in spleen by immunoblotting, although HaPrP mRNA was detected at low levels.

Inoculation of Tg3/*Prnp*^{0/0} mice with hamster scrapie agent resulted in neurological disease after a relatively long incubation time of 227 days. This was not unexpected in view of the dependence of incubation time on PrP^C expression levels (Prusiner *et al.*, 1990; Büeler *et al.*,

1994; Manson *et al.*, 1994b; Fischer *et al.*, 1996). More surprising was the rapid progression of the clinical phase leading to death of all animals within about a week. This might be explained by the inducible nature of the GFAP-HaPrP transgene leading to increased levels of HaPrP^C after astrocyte activation, thereby providing more substrate for the conversion of HaPrP^C into HaPrP^{Sc}.

In the present experiments, expression of mouse PrP in GFAP-HaPrP transgenic mice completely prevented clinical disease, and diminished HaPrP^{Sc} formation and propagation of hamster scrapie agent. Similar competitive or inhibitory effects between coexpressed PrP molecules

of different species have been noted previously both in transgenic mice (Scott *et al.*, 1989, 1992; Telling *et al.*, 1996) and in mouse neuroblastoma cell cultures (Scott *et al.*, 1992; Priola *et al.*, 1994; Priola and Chesebro, 1995; Race *et al.*, 1995). These results indicate, once more (Büeler *et al.*, 1994; Manson *et al.*, 1994b), that agent propagation to high levels is not necessarily sufficient to cause pathology or clinical symptoms.

In the course of the disease in wild-type mice, PrP^{Sc} has been found to accumulate in astrocytes prior to the development of neuropathological changes (Diedrich *et al.*, 1991), suggesting that PrP^{Sc} released into the extracellular compartment (Jeffrey *et al.*, 1994) might contribute to pathogenesis by inherent toxicity. However, as shown recently (Brandner *et al.*, 1996), PrP^{Sc} was unable to elicit a neurotoxic effect when presented to PrP^{0/0} neural tissue; neurotoxicity only resulted when cells were PrP^{+/+} and thus able to produce PrP^{Sc} endogenously. *Tg3/Prnp^{0/0}* mice express HaPrP^C in astrocytes but not in neurons, and therefore in these animals only astrocytes should be susceptible to direct pathological effects mediated by PrP^{Sc}. However, because astrocytes are involved in maintaining the normal status of neurons (Mucke and Eddleston, 1993), astrocyte dysfunction might result in neuronal abnormalities. Interestingly, onset and development of neuropathological changes in scrapie correlate with increased expression of a variety of cytokines (Campbell *et al.*, 1994; Williams *et al.*, 1994). Such cytokines might be produced by astrocytes or other glial cells, and might play a key role in the pathological events of scrapie via indirect effects leading to neuronal dysfunction, analogous to that which has been seen following infection of glial cells by murine retroviruses which cause a spongiform pathology quite like scrapie (Lynch *et al.*, 1991).

Our findings that astrocytosis and spongiosis in the terminal phase of scrapie are quite similar in wild-type mice and in mice expressing PrP in either neurons or astrocytes only, suggest that a common final pathway, perhaps cytokine overproduction, contributes to the neuropathological picture in all these cases.

Materials and methods

Generation of transgenic mice

A 1 kb fragment of hamster PrP cDNA containing the open reading frame was cloned into pBluescript-KS⁺ to generate plasmid p2-17, as described previously (Race *et al.*, 1995). A *NorI* linker was added at the *KpnI* site in the polylinker and a 1.2 kb fragment was excised with *NorI* and inserted in place of the *lacZ* gene in clone C-445 containing a modified GFAP gene (Johnson *et al.*, 1995). Digestion of this plasmid with *SfiI* to remove the vector sequences yielded the DNA fragment used to generate GFAP-HaPrP transgenic mice. Microinjection into pronuclei of fertilized oocytes was according to standard procedures. Transgene-positive founders were identified by semiquantitative dot blot hybridization and further characterized by Southern analysis of tail DNA, using an HaPrP DNA probe. Five lines (*Tg3* to *Tg7*) had about two to four transgene copies. *Tg3* mice were crossed with *Prnp^{0/0}* mice (Büeler *et al.*, 1992) in Zürich and with *Prnp^{-/-}* mice (Manson *et al.*, 1994a) at RML; F₃ PrP knockout progeny carrying the hamster PrP transgenes were identified by hamster-specific PCR on tail DNA. In Zürich, two separate PCRs were performed; the mouse *Prnp⁰* and *Prnp⁺* alleles were detected by the three primers RK1, RK2 and RK3 (Prusiner *et al.*, 1993), and the hamster PrP transgene was amplified using the two primers GFAP1 (5')AACCGTTACCCACCTCAGGGT and GFAP2 (5')CTCCT-TCTGATACCTGGTGGT to yield a 521 bp product. The reactions were performed in 25 µl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM dNTP, 50 µM of each

primer and 0.1 units SuperTaq DNA polymerase. Amplification was carried out by cycling 35 times for 1 min at 94°C, 1 min at 60°C and for 45 s at 72°C. At RML, three reactions were used. One of the reactions identified mice positive for HaPrP. The oligonucleotide sequences and reaction conditions have been described (Race *et al.*, 1995). A second set of oligonucleotide primers specific for a portion of the neomycin resistance gene was used to identify mice containing the disrupted non-functional mouse (Mo) PrP gene (3'-CTTGGACGCACGTTAGGTAG-5') and (5'-TTGAGCCTGGCGAACAGTTC-3'). The third set of primers, specific for MoPrP, distinguished mice heterozygous for the disrupted MoPrP from those homozygous for the disrupted MoPrP gene (5'AACCGTTACCCACCTCAGGGT-3') and (3'-ACACTTCTACTACTCTCGCG-5'). The same conditions were used for all three PCRs.

Sources of infectious scrapie agent

RML (Chandler, 1961) is a mouse-adapted scrapie isolate which was passaged in Swiss CD-1 mice obtained from Charles River Laboratories. The Syrian hamster-adapted isolate of scrapie strain 263K (Kimberlin and Walker, 1978) was passaged repeatedly in Golden Syrian hamsters. The Sc237 isolate (Scott *et al.*, 1989) was provided by S.B. Prusiner.

Scrapie infection and diagnosis

Hamsters and mice were inoculated i.c. with 50 and 30 µl, respectively, of a 1% brain suspension of scrapie-infected hamster brain. Brain homogenates were made from several of the *Tg3/Prnp^{0/0}* and *Tg3/Prnp^{+/+}* mice at different times after inoculation. To titrate the levels of infectivity, 50 µl of various dilutions of these brain homogenates were inoculated i.c. into weanling Syrian hamsters which were then inspected two or three times per week for the appearance of scrapie symptoms.

Northern analysis

For the experiment in Figure 1C, brain poly(A)⁺ RNA was isolated using a micro Fast-Track kit (Invitrogen) and was analyzed as previously described (Race *et al.*, 1995). For Figure 4C, total brain RNA was prepared as described previously (Chomczynski and Sacchi, 1987), and poly(A)⁺ RNA was isolated using oligo-(dT) latex beads (Qiagen) according to the manufacturer's instructions. Poly(A)⁺ RNA was denatured, electrophoresed through 2.2 M formaldehyde-1% agarose gels and blotted onto Hybond-N+ (Amersham) membranes in 20× SSC. Prehybridization and hybridization were performed with Quickhyb (Stratagene) according to the manufacturer's instructions. The membranes were probed with the 256 bp *KpnI*-*Bst*EII fragment of the hamster PrP ORF which was ³²P-labeled by the random primer method (Prime-It, Stratagene). GFAP mRNA was detected with a mouse GFAP cDNA probe (a kind gift from U. Pott, Brain Research Institute, University of Zürich). For glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA as an internal standard, the 490 bp *XhoI* fragment from rat GAPDH subcloned into pSP64 was used as a probe (Fort *et al.*, 1985).

Western blot analysis

For Figure 1B and D and Figure 4B, proteins were analyzed by immunoblot as previously described (Race *et al.*, 1995). For Figure 4A, 10% brain homogenates were prepared in PBS containing 0.5% NP40 and 0.5% sodium deoxycholate by passing the brains through 18-gauge and 22-gauge needles. The homogenate was centrifuged at 1500 g for 10 min, and the supernatant was adjusted to 8 mg/ml of total protein using the BCA protein assay according to the manufacturer's instruction (Pierce). Where indicated, aliquots of the homogenate were digested with 20 µg/ml proteinase K for 30 min at 37°C, and the reactions were stopped by adding PMSF to 2 mM and boiling in SDS-PAGE loading buffer without β-mercaptoethanol. The samples (80 µg of total protein) were electrophoresed through 12% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and probed with monoclonal antibody to hamster PrP [3F4, 1:10000 (Kascsak *et al.*, 1987)], developed using the enhanced chemiluminescence kit (Amersham) and exposed to Kodak X-ray film.

In situ hybridization

In situ hybridization for PrP RNA was performed on selected brain sections. The 290 bp (*Asp*718-*Bst*EII) fragment of mouse PrP cDNA was cloned into pBluescript KS and SK vector (Stratagene, La Jolla, CA), respectively. Digoxigenin-labeled sense and antisense probes were synthesized (Boehringer, Mannheim) from the *Asp*718-cleaved KS and SK constructs, respectively, using T3 RNA polymerase. Sections were deparaffinized and postfixed in 4% paraformaldehyde in PBS, mildly digested with proteinase K (10 µg/ml for 5 min at 37°C) and permeabilized for 10 min in 0.1 M HCl. After a second fixation step in 4%

paraformaldehyde for 10 min, acetylation was performed in triethanolamine 0.1 M, acetic anhydride 0.25% and prehybridization was carried out in $5\times$ SSC, 50% formamide, $5\times$ Denhardt's and 250 μ g/ml yeast tRNA for 3–5 h at room temperature. Hybridization was in the same mixture containing the digoxigenin-labeled probe at 200–400 ng/ml for 12 h at 65°C. The sample was washed for 1 h at 65°C in $0.2\times$ SSC, labeled with anti-DIG Fab fragments (1:500) and developed with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitroblue tetrazolium chloride following the supplier's protocol. For subsequent GFAP immunolabeling, sections were heated to 80°C to inactivate alkaline phosphatase and processed as described below. The sections were mounted in glycerol–gelatin.

Immunohistochemistry

For paraffin histology, whole mouse brains were fixed for at least 24 h in 4% paraformaldehyde in phosphate-buffered saline (PBS), immersed for 1 h in 98% formic acid and postfixed for 72 h in 4% paraformaldehyde/PBS. Coronal slices of ~2 mm thickness were dehydrated through graded alcohols and embedded in paraffin. Sections of 4 μ m nominal thickness were mounted on TESPA (SIGMA Chemicals)-coated glass slides and routinely stained with hematoxylin and eosin (H&E). Immunohistochemistry for GFAP [polyclonal, 1:300 (Dako, Glostrup, DK)] was performed on paraffin-embedded sections. Biotinylated secondary antibodies (goat anti-rabbit; DAKO) were used at a dilution of 1:200. Visualization was achieved using biotin/avidin-peroxidase (DAKO) and diaminobenzidine as a chromogen following the protocols suggested by the manufacturer. Alkaline phosphatase-labeled secondary antibodies (rabbit anti-mouse, DAKO) were used at a 1:30 dilution and visualized by the new fuchsin color reaction according to manufacturer's protocol.

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